WEST Search History

DATE: Monday, January 06, 2003

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT; PLUR=YES; OP=ADJ			
L4	laccase and ((800/288)!.CCLS.) and commercial near production	0	L4
L3	laccase and ((800/288)!.CCLS.) and trametes and (corn or maize)	0	L3
DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L2	L1 and (corn or maize)	43	L2
L1	laccase and transgenic near plant	46	L1

END OF SEARCH HISTORY

WEST

End of Result Set

Generate Collection Print

WO 97/45549

L2: Entry 43 of 43 File: DWPI Dec 4, 1997

DERWENT-ACC-NO: 1998-032655
DERWENT-WEEK: 199821

TITLE: Modifying lignin biosynthesis in plants with gene encoding laccase mRNA - or its anti-sense complement, especially for crops used as fodder and for paper production

Basic Abstract Text (1):
Use of recombinant nucleic acid sequence (I) to transform plant cells for producing transgenic plants having a reduced content of lignin (II) and/or altered composition of lignins is new. (I) contains at least one coding region which: (i) is a (fragment of a) sequence that produces a mRNA encoding a laccase (III), or a derivative of such a sequence (particularly one having at least one nucleotide mutated, added, deleted or substituted) encoding a (III) derivative; or (ii) is a(fragment of a) sequence complementary to the sequence of (i) encoding an antisense mRNA able to hybridise to (III)-encoding mRNA in the plant.

Basic Abstract Text (2):
USE - Sequences expressing laccase mRNA (sense or antisense) are used to produce plants of reduced (II) content while sense sequences may also be used to increase (II) biosynthesis. Particularly the method is applied to (i) forage crops (lucerne, silage maize etc.) to improve digestibility or (ii) to plants used for paper manufacture (so that lignin extraction is easier and less polluting). Plants with increased (II) content may have better resistance to parasites and greater value as fuels. Those with reduced (II) content may provide better growth and yield. Also (not claimed) antibodies (Ab) raised against laccase can be used to detect and/or determine laccase levels.

Standard Title Terms (1):
MODIFIED LIGNIN BIOSYNTHESIS PLANT GENE ENCODE LACCASE MRNA ANTI SENSE COMPLEMENTARY CROP FODDER PAPER PRODUCE

More particularly, the goal of this invention is to give a method for efficient control of plant lignin content, either in order to significantly reduce such content as compared to normal plant content, or in order to increase such content.

Another goal of this invention is to supply the tools for the implementation of such method, and more particularly constructions that may be used for plant transformation.

Another goal of this invention is to supply genetically transformed plants, especially feed plants liable to be better digested than non-transformed plants, or plants or trees transformed for paper pulp production, in which

WO 97/45549

lignin extraction is easier and less polluting than in non-transformed trees.

Another goal of this invention is to supply transformed plants which are more resistant to environmental attacks, especially parasite attacks, than non-transformed plants or larger transformed plants, or smaller transformed plants (than non-transformed plants).

The invention is illustrated with the help of figures 1 through 5 below:

- figure 1: representation of the construction of plasmid pBKTL8, designed to over-express a gene coding for a laccase; it was made based on SEQ ID NO 1, i.e., based on a complete sequence of cDNA coding a tobacco laccase (coding sequence and 5' and 3' ends non-coding for cDNA).

- figure 2: representation of the construction of plasmid pMBKTL corresponding to the plasmid pBKTL8 presented in figure 1, in which the NcoI restriction site located upstream from the sequence coding the tobacco laccase, has been eliminated.

- figure 3: representation of construction of plasmid pNKTL, designed to over-express a gene coding for a laccase; it was made based on a complete sequence of cDNA coding a tobacco laccase delimited by the nucleotides located in positions 82 and 1755 of SEQ ID NO 1 (cloning of the coding region only).

- figure 4: representation of the construction of plasmids pHS1 and pHS16, designed to reduce the expression of the gene(s) coding for laccase(s); they were obtained based on a partial cDNA sequence coding a tobacco laccase, i.e., the sequence delimited by the nucleotides located in positions 292 and 1766, which was cloned in sense direction in the case of plasmid pHS16, and in antisense direction in the case of plasmid pHS1.

- figure 5: representation of the construction of plasmid pES22, designed to reduce the expression of the gene(s) coding for laccase(s); it was obtained based on SEQ ID NO 1, i.e., based on a complete sequence of cDNA coding a tobacco laccase (coding sequence and 5' and 3' ends non-coding for cDNA), which was cloned in antisense direction.

- the nucleotide sequence represented by SEQ ID NO 1, coding for a mRNA, such mRNA itself coding for the tobacco laccase represented by SEQ ID NO 2.

- the nucleotide sequence represented by SEQ ID NO 3, coding for a mRNA, such mRNA itself coding for a fragment of a tobacco laccase, such fragment being represented by SEQ ID NO 4.

- the nucleotide sequence represented by SEQ ID NO 5, coding for a mRNA, such mRNA itself coding for a fragment of a tobacco laccase, such fragment being represented by SEQ ID NO 6.

- the nucleotide sequence complementary to that represented by SEQ ID NO 1, SEQ ID NO 3, or SEQ ID NO 5, such complementary sequence coding for an antisense mRNA, capable of being hybridized with an mRNA coding for a plant laccase and more particularly with the mRNA coded by sequences SEQ ID NO 1, SEQ ID NO 3, and SEQ ID NO 5, respectively.

- the nucleotide sequence derived from the sequence represented by SEQ ID NO 1, SEQ ID NO 3, or SEQ ID NO 5, especially by mutation and/or addition, and/or suppression, and/or substitution of one or several nucleotides, such derived sequence coding either for an mRNA which codes itself for the laccase represented by SEQ ID NO 2, or for the fragments of laccase represented by SEQ ID NO 4 or SEQ ID NO 6, or for a protein derived from the above-mentioned laccase or fragments of laccase.

For this reason, more particularly, the goal of the invention is a method to reduce the quantity of lignins produced by biosynthesis in plants, such method being embodied by transformation of the genome of these plants, incorporating:

- at least one DNA sequence according to the invention, represented by SEQ ID NO 1, SEQ ID NO 3, or SEQ ID NO 5, or a fragment or sequence derived from the latter, as defined above

- and, if applicable, at least one DNA sequence coding for all or part of an enzyme other than the laccase, which is involved in a stage of the biosynthesis of plant lignins, especially a DNA sequence coding for all or part of CAD, or CCR, or OMT,

said transformation being obtained:

- either with the help of a recombinant vector such as that described above, containing the DNA sequence according to the invention, as mentioned above, or a fragment or sequence derived from the latter, as defined above and, if applicable, containing one or several sequence(s) of DNA coding for all or part of an enzyme other than laccase, as defined above.

- or with the help of several recombinant vectors, of which at least one contains a DNA sequence according to the invention, as mentioned above, or a fragment or sequence derived from the latter, as defined above, while the other recombinant vector(s) contain(s) a DNA sequence coding for all or part of an enzyme other than laccase, as defined above.

It is noteworthy that the above-mentioned methods allow obtaining transformed plants with different levels of reduction in laccase activity (depending on the level of insertion of the DNA sequence coding for the antisense mRNA, the number of copies of this DNA sequence integrated in the genome...), and therefore lignin contents.

The choice of transformers will allow for a controlled modulation of lignin contents compatible with normal plant development.

In general, if it is considered that the normal average lignin content of a plant varies between approximately 15% and approximately 35% in dry material weight, the reduction of lignin content resulting from the implementation of one of the above-mentioned methods is advantageously such that the plants so transformed have an average lignin content reduced by at least approximately 2 to 3% as compared to the normal average lignin content of a non-transformed plant.

1) Cloning of cDNA coding for tobacco laccase

A bank of cDNA originating from tobacco stems (*Nicotiana tabacum* cv Samsun), built in the EcoRI site of the vector λ ZAPII (Stratagene, Cambridge, U.K.) was cleaved with sycamore laccase, with the help of a heterologous probe. This sequence corresponds to the sequence of a cDNA with 435 basic pairs (bp) published by Delaunay et al. (1994), coding for part of the above-mentioned laccase, and covering the 3' end of the cDNA sequence (including a zone coding for a protein with two preserved copper fixation regions) published by La Fayette et al. (1995).

About 400,000 lysis sites were cleaved and four positive clones were detected. After excision of the λZAPII phage in pBluescript (SK-) plasmid *in vivo*, these clones were examined.

The approximate size of the insert of the four clones (pTL1-4) varies by approximately 700 bp to 2,000 bp.

A preliminary analysis of these clones with the help of restriction enzymes showed that two of them (pTL1 and pTL3) contain the same insert (also confirmed by the partial sequencing of these clones).

The three clones pTL2, pTL3 and pTL4 were further characterized by sequencing.

Among the three clones analyzed, the one with the largest insert, namely pTL3, contains a cDNA coding for a complete laccase. The PTL3 insert has a length of 1984 bp (SEQ ID NO 1) and is made up of a non-translated region of 81 bp in 5', an open reading phase coding for a laccase of 1674 bp, and a non-coding region of 229 bp in 3'.

The other two cDNA of the clones pTL2 and pTL4 have a length of 1512 bp (SEQ ID NO 2) and 630 (SEQ ID NO 3), respectively, and correspond to partial sequences which do not contain the region coding for the N-terminal part of the laccase. However, clones pTL2, -3 and -4 contain cDNA coding for different laccases, since their sequences are different in overlapping regions. The deducted sequence in amino acids of the nucleotide sequence of the insert of pTL3 is identical to 89% of the amino acid sequences derived from pTL2 and pTL4, which present 95% identity between themselves. The three cDNA sequences also have different non-coding regions in 3'.

The cDNA insert of pTL3 codes for a polypeptide of 557 amino acids (61.9 kDa) with an isoelectric point (Ip) of 10.08.

The deduced protein has twelve possible N-glycosylation sites (Asn-Xaa-Ser/Thr). The N-terminal hydrophobic region of 22 amino acids probably represents the signal peptide, as suggested by a comparison with the sycamore laccase sequence. The scission of this signal peptide must take place between the residues Lys in position 22 and Arg in position 23, according to the rules of Von Heijne (Von Heijne, 1986). Consequently, the mature protein contains 535 amino acids (59.4 kDa).

The comparison of the entire amino acid sequence deduced from the sequence of pTL3 with the amino acid sequence deduced from sycamore laccase (*Acer pseudoplatanus*) (La Fayette et al., 1995), indicated that these two proteins have 48% identity, such identity being even higher (60%) when considering the 100 C-terminal amino acids.

This C-terminal region contains two potential copper fixation sites.

The sequence of the tobacco laccase was also compared to other copper oxydases found in data banks. Among these proteins, those with the greatest homology are cucumber and tobacco ascorbate oxydases, with 36% and 34% identity, respectively, by using the BESTFIT programs (Genetics Computer Group, Wisconsin Package, Version 8).

Similar comparisons were made with fungal laccases: 31% and 28% identity in amino acids

were found with the sequences deducted, respectively, from *Cryptococcus neoformans* and *Neurospora crassa*.

In each case, regions kept in the peptide sequence of the tobacco laccase were identified, these regions being very similar to the copper fixation sites kept among copper oxydases (Ohkawa et al., 1989; Messerschmidt and Huber, 1990).

2) Construction of plasmids containing nucleotide sequences under the invention

The plasmid pBKTL8 (see figure 1) was designed to over-express a gene coding for a laccase; it was obtained based on SEQ ID NO 1, i.e., from a complete sequence of cDNA coding a tobacco laccase (coding sequence and non-coding cDNA ends 5' and 3').

The plasmid pMBKTL (see figure 2), corresponding to the pBKTL8 plasmid presented in figure 1, in which the NcoI restriction site located upstream from the sequence coding the tobacco laccase, was eliminated.

The plasmid pNKTL (see figure 3) was designed to over-express a gene coding for a laccase; it was obtained based on the cDNA sequence coding a tobacco laccase delimited by the nucleotides located in positions 82 and 1755 of SEQ ID NO 1 (cloning of the coding region only).

Plasmids pHS1 and pHS16 (see figure 4) were designed to reduce the expression of the gene(s) coding for laccase(s); they were obtained based on a partial cDNA sequence coding a tobacco laccase, i.e., the sequence delimited by the nucleotides located in positions 292 and 1766, which was cloned in sense direction in the case of plasmid pHS16, and in antisense direction in the case of plasmid pHS1.

Plasmid pES22 (see figure 5) was designed to reduce the expression of the gene(s) coding for laccase(s); it was obtained based on SEQ ID NO 1, i.e., based on a complete sequence of cDNA coding a tobacco laccase (coding sequence and 5' and 3' ends non-coding for cDNA), which was cloned in antisense direction.

The details for the construction of these plasmids is indicated in the legends of figures 1 and 5 below.

FIGURE LEGENDS

- Figure 1: Preparation of plasmid pBKTL8

a) Schematic representation of the cDNA fragment contained in plasmid pTL3

The sequence of cDNA coding for a tobacco laccase is indicated in white. Ends 5' and 3' non-coding for cDNA are indicated in black. The insertion of cDNA was freed by digestion by restriction enzymes SmaI and KpnI.

b) Schematic representation of the expression box of the cloning vector pMJBx

CaMV 35S: sequences increasing the transcription ("enhancer") and promoter of the RNA 35S of the cauliflower mosaic virus (CaMV); [symbol]: omega element of the tobacco mosaic virus (TMV); nos3': region of the end of the gene coding nopaline synthase; the multi cloning site is indicated in black.

c) Schematic representation of the expression box contained in plasmid pBK1

Plasmid pBK1 was obtained by cloning of the fragment of cDNA fragment SmaI-KpnI originating from pTL3 (see a) in plasmid pMJBx (see b), digested by restriction enzymes BamHI (digestion followed by treatment by the Klenow fragment of DNA polymerase 1, in order to generate an end with free tips) and KpnI.

d) Schematic representation of the restriction fragments obtained from plasmid pBK1, used for cloning in the binary vector.

- after digestion by the enzymes HindIII and XbaI (at the top)

- after digestion by the enzyme XbaI (at the bottom)

e) Schematic representation of the T-DNA region of the binary vector pBinI9i

RB: right border of T-DNA; nos promoter: sequence promoter of the gene coding nopaline synthase; nptII CDS: sequence coding the kanamycin-resistance gene; nos3': ending sequence of the gene coding nopaline synthase; LB: left border of T-DNA; the multi cloning site is indicated in black.

f) Schematic representation of the T-DNA region of the pBKTL8 transformation vector

The pBKTL8 transformation vector was obtained by cloning of the expression box contained in plasmid pBK1 (see c) in the binary vector pBin19i (see e). This cloning was done in two stages:

the HindIII-Xbal fragment originating from pBK1 (see d) was first inserted in the plasmid pBin19i digested by enzymes HindIII and Xbal, then the resulting plasmid was linearized by digestion by the enzyme Xbal, in order to clone the XbaI-XbaI fragment in a second step (see d).

- Figure 2: Preparation of plasmid pMBKTL

a) Schematic representation of the expression box contained in plasmid pBK1

The plasmid pBK1 was obtained by cloning the fragment of cDNA SmaI-KpnI originating from pTL3 in the plasmid pMJBx (see figure 1c). The undesirable restriction site NcoI is indicated.

b) Schematic representation of the expression box contained in the plasmid pMBK41

The plasmid pMBK41 derives from the plasmid pBK1, which was modified in order to eliminate the NcoI restriction site. To obtain the plasmid pMBK41, the plasmid pBK1 was linearized by digestion by the enzyme NcoI, then the NcoI restriction site was destroyed by digestion by the Mung Bean nuclease, and the plasmid was closed on itself. The position of the eliminated NcoI site is indicated.

c) Schematic representation of the expression box contained in plasmid pMBK41

Plasmid pMBK41 was digested by the restriction enzyme XhoI in order to free a fragment corresponding to the omega sequences and that of the cDNA coding tobacco laccase (see d). The XhoI sites are indicated.

d) Schematic representation of the restriction fragments obtained by digestion of the plasmid pMBK41 by the XhoI enzyme, and used for cloning in the binary vector

e) Schematic representation of the T-DNA region of the transformation vector pBKTL8

The construction of the plasmid pBKTL8 was described in figure 1. The restriction sites XhoI present in this plasmid are indicated. The NcoI site is also indicated. pBKTL8 was digested by the enzyme XhoI, in order to eliminate a fragment corresponding to the omega sequences and that of the cDNA of tobacco laccase, and to replace it by the equivalent region which no longer contains the restriction site NcoI, and originating from pMBK41 (see c and d). The following chart (f) presents the new plasmid obtained.

f) Schematic representation of the T-DNA region of the pMBKTL transformation vector

The pMBKTL transformation vector was obtained by replacing a restriction fragment XhoI-XhoI containing a region corresponding to the omega sequences and that of the cDNA coding the tobacco laccase of the pBKTL8 plasmid (see e) by an equivalent fragment originating from plasmid pMBK41 (see c and d). pMBKTL differs from pBKTL8 only by the absence of a NcoI restriction site.

- Figure 3: Preparation of the plasmid pNKTL

a) Schematic representation of the cDNA fragment contained in the plasmid pTL3 and the primers used to enhance the coding sequence through PCR

The sequence of cDNA coding for a tobacco laccase is indicated in white. Ends 5' and 3' non-coding for cDNA are indicated in black. The position of the primers used to enhance through PCR the region corresponding to the sequence coding the tobacco laccase is indicated. These primers were defined so as to allow introducing NcoI restriction sites (at the level of the ATG codon at the beginning of traduction) and KpnI at the ends 5' and 3', respectively, of the PCR enhancement product.

b) Schematic representation of the PCR enhancement product obtained from plasmid pTL3

c) Schematic representation of the expression box of the cloning vector pMJB1

CaMV 35S: "enhancer" sequences and promoter of the RNA 35S of the cauliflower mosaic virus (CaMV); [symbol]: omega element of the tobacco mosaic virus (TMV); nos3': region of the end of the gene coding nopaline synthase; the multi cloning site is indicated in black.

d) Schematic representation of the expression box contained plasmid pNK8

The plasmid pNK8 originates from the cloning of the PCR enhancing product obtained from pTL3, digested by restriction enzymes NcoI and KpnI (see a and b), and inserted in the plasmid pMJB1 (see c) digested by the same enzymes.

Most of the sequences originating from the PCR enhancement were then eliminated from the plasmid pNK8 in order to avoid the presence of possible sequence errors. This was done by replacing the region corresponding to the restriction fragment obtained by digestion by the enzymes BsmI and XbaI with the equivalent region of the original plasmid pTL3 (see e).

e) Schematic representation of the cDNA fragment contained in plasmid pTL3

The sequence of cDNA coding for a tobacco laccase is indicated in white. Ends 5' and 3' non-coding for cDNA are indicated in black. The position of the restriction sites BsmI and XbaI is indicated.

f) Schematic representation of the expression box of the contained in plasmid pBx3

Plasmid pBX3 was obtained from plasmid pNK8, by replacing a region delimited by the restriction sites BsmI and XbaI with the equivalent region of the original plasmid pTL3. The regions arising from PCR enhancement are indicated in black, and were verified by sequencing, while the sequences of the original cDNA are represented in white.

g) Schematic representation of the expression box contained in plasmid pBX3 and restriction fragments used for cloning in the binary vector

The regions arising from PCR enhancement are indicated in black, while the sequences of the original cDNA are represented in white.

In order to clone the expression box contained in plasmid pBX3 in the binary vector pJR1Ri, pBX3 was digested by restriction enzymes HindIII and EcoRI, generating two restriction fragments: HindIII-EcoRI, and EcoRI-EcoRI, which were cloned in two stages in the binary vector.

h) Schematic representation of the T-DNA region of the binary vector pJR1Ri

LB: left border of T-DNA; nos 3': region of the end of the gene coding nopaline synthase; CaMV 35S: sequences increasing the transcription ("enhancer") and promoter of the RNA 35S of the cauliflower mosaic virus (CaMV); nptII CDS: coding sequence of the kanamycin-resistance gene; nos promoter: promoter sequence of the gene coding nopaline synthase; RB: right border of T-DNA.

i) Schematic representation of the T-DNA region of the pNKTL transformation vector

The pNKTL transformation vector was obtained by cloning of the expression box contained in plasmid pBX3 (see g) in the binary vector pJR1Ri (see h). This cloning was done in two stages: the HindIII-EcoRI fragment originating from pBX3 (see g) was first inserted in the plasmid pJR1Ri digested by enzymes HindIII and EcoRI, then the resulting plasmid was linearized by digestion by the

enzyme EcoRI, in order to clone the EcoRI-EcoRI fragment in a second step (see g).

- Figure 4: Preparation of plasmids pHS1 and pHS16

a) Schematic representation of the cDNA fragment contained in plasmid pTL3

The sequence of cDNA coding for a tobacco laccase is indicated in white. Ends 5' and 3' non-coding for cDNA are indicated in black. A fragment of cDNA corresponding to a partial cDNA sequence coding for a tobacco laccase was obtained by digestion of the plasmid pTL3 by restriction enzymes HindII and StuI. The position of these restriction sites is indicated.

b) Schematic representation of the restriction fragment obtained by digestion of the plasmid pTL3 by enzymes HindII and StuI, and used for cloning in the binary vector

c) Schematic representation of the T-DNA region of the binary vector pJR1Ri

RB: right border of T-DNA; nos promoter: sequence promoter of the gene coding nopaline synthase; nptII CDS: sequence coding the kanamycin-resistance gene; nos3': ending sequence of the gene coding nopaline synthase; CaMV 35S: "enhancer" sequences and promoter of the RNA 35S of the cauliflower mosaic virus (CaMV); LB: left border of T-DNA; the cloning site is indicated in black.

d) Schematic representation of the T-DNA region of the pHS16 transformation vector

The pHS16 transformation vector was obtained by cloning of the HindII-StuI cDNA fragment originating from pTL3 (see a and b) in the binary vector pJR1Ri (see c), linearized by digestion by the restriction enzyme SmaI. The cDNA sequence is inserted in sense direction.

e) Schematic representation of the T-DNA region of the pHS1 transformation vector

The pHS1 transformation vector was obtained by cloning of the HindII-StuI cDNA fragment originating from pTL3 (see a and b) in the binary vector pJR1Ri (see c), linearized by digestion by the restriction enzyme SmaI. The cDNA sequence is inserted in antisense direction.

- Figure 5: Preparation of plasmid pES22

a) Schematic representation of the cDNA fragment contained in plasmid pTL3

The sequence of cDNA coding for a tobacco laccase is indicated in white. 5' and 3' Ends non-coding for cDNA are indicated in black. The insertion of the cDNA was freed by digestion by restriction enzymes SmaI and EcoRV.

b) Schematic representation of the T-DNA region of the binary vector pJR1Ri

RB: right border of T-DNA; nos promoter: sequence promoter of the gene coding nopaline synthase; nptII CDS: sequence coding the kanamycin-resistance gene; nos3': ending sequence of the gene coding nopaline synthase; CaMV 35S: "enhancer" sequences and promoter of the RNA 35S of the cauliflower mosaic virus (CaMV); LB: left border of T-DNA; the cloning site is indicated in black.

c) Schematic representation of the T-DNA region of the pES22 transformation vector

The pES22 transformation vector was obtained by cloning of the SmaI-EcoRV cDNA fragment originating from pTL3 (see a) in the binary vector pJR1Ri (see b), linearized by digestion by the restriction enzyme SmaI. The cDNA sequence is inserted in antisense direction.